R. Bras. Zootec., 52:e20220137, 2023
https://doi.org/10.37496/rbz5220220137

Breeding and genetics
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
e-ISSN 1806-9290
www.rbz.org.br

RNA-Seq study of *in vivo*-produced single male and female bovine blastocysts

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ABSTRACT - The objective of this study was to identify differentially expressed genes (DEG) between females and males in early bovine embryos. Superovulated cattle (n = 4) in the age span of 16–18 months were artificially inseminated with semen from the same bull that has been proven to be fertile. Blastocysts were collected by routine non-surgical uterine flushing on day 7 after insemination. This study determines the sex of embryos using a micro-injection pipette to collect a few blastomeres through the Zona pellucida. The remaining blastomeres were used for single-cell RNA sequencing (scRNA-seq) analysis on the Illumina platform, followed by differential expression analysis, Gene Ontology (GO) function analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The criteria for identifying DEG will be set at |log2(Fold change)| ≥ 1 and P<0.05. A total of 8004 DEG were detected in female (Denoted as BLX, n = 3) and male (Denoted as BLY, n = 3) blastocysts. Transcripts highly expressed in the female embryos were related to catalytic activity, nucleotide binding, and catabolic process, while transcripts highly expressed in the male embryos were linked to oxidative phosphorylation, mitochondrion, and the ribosome. Nine genes that may be involved in blastocyst growth and development were screened, suggesting that their differential expression may be responsible for the differences in the development of male and female embryos. This study provides important information on potential genes and pathways associated with differences in early female and male embryonic development.

Keywords: bovine pre-implantation embryos, differential gene expression, gender, transcriptome

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Received: October 9, 2022
Accepted: August 11, 2023

How to cite: Cui, B.; Wang, J.; Huang, F.; Li, N. and Gao, Q. 2023. RNA-Seq study of *in vivo*-produced single male and female bovine blastocysts. Revista Brasileira de Zootecnia 52:e20220137. https://doi.org/10.37496/rbz5220220137

1. Introduction

Numerous studies have highlighted the difference in the pace of embryonic pre-implantation period development between female and male mammalians (Inaba et al., 2016). These findings suggest developmental inconsistencies exist between male and female mammals long before gonads are identified and developed (Erickson, 1997; Laguna-Barraza et al., 2013). Since these differences precede the production of gonadal steroid hormones in both sexes, hormonal influences cannot explain these phenomena and must be elucidated genetically.

Studies from different species show differences in growth rate and metabolism between male embryos cultured *in vitro* and female embryos of the same stage (Nedambale et al., 2004). In human pre-implantation, male embryos show higher cell counts than female embryos from when cultured on day 2 until the blastocyst stage (Ray et al., 1995). These differences may be caused

by the *in vitro* culture conditions (Nedambale et al., 2004; Inaba et al., 2016). Although *in vitro* culture systems have been well established and have been used by many laboratories for decades (Watson et al., 2000), it has also been reported that blastocysts produced *in vitro* have different transcriptomic profiles than those produced *in vivo* (Smith et al., 2009; Driver et al., 2012). The rate of metabolism, transcript abundance differences, and mitochondrial activity may also be other factors contributing to differences in embryonic development between males and females (Acton, 2004; Bermejo-Álvarez et al., 2008). So far, there has been no clear answer as to whether transcriptional differences between male and female embryos influence early embryonic (during embryonic development, pre-implantation embryos are generally called early embryos) development (Capela, 2022).

The recent developments in high-throughput sequencing technologies allow for gene expression analysis of individual embryos and blastomeres. scRNA-seq techniques can overcome the difficulties of limited total ribonucleic acid (RNA) content in embryos and sparse embryo samples (Pérez-Palacios et al., 2021). Jiang et al. (2014) applied the RNA-seq platform on single *in vivo* matured oocytes and *in vivo* developed embryos and obtained their transcriptome data. Later, Diaz-Lundahl et al. (2022) performed RNA-seq research on *in vivo*-produced single embryos from Norwegian red bulls with high or low non-return rates, and the genetic contribution of males during embryonic development was revealed. Therefore, scRNA-seq has become the method of choice to study transcriptional differences in embryos of both sexes.

Bovine male blastocysts develop faster than female blastocysts, which is related to transcriptional differences between sex embryos. To perform this study, we obtained sex-preselected bovine *in vivo*-derived blastocysts by built-in control nested polymerase chain reaction (PCR) and performed scRNA-seq analysis on the Illumina platform. This study aimed to analyze the differences in mRNA levels between female and male bovine blastocysts. These findings will contribute to a better understanding of the relevant molecular regulatory mechanisms underlying pre-implantation sex differences.

2. Material and Methods

2.1. Animals

Research on animals was conducted according to the institutional committee on animal use (2016000605TD). The experiments were performed in Alar, Xinjiang, China (40°54' N, 81°30' E). We used four female Holstein cattle, 16 to 18 months of age, as embryo donors, which were kept in a wide playground with sand bedding and had *ad libitum* access to ration and water. They were further divided into treatment groups and control groups according to experimental requirements.

2.2. Production of *in vivo* blastocysts

Holstein cattle that were confirmed by rectal examination to be unpregnant and had a normal reproductive tract and ovarian function were synchronized and superovulated as described in Hayakawa et al. (2009) and Lee et al. (2012). Artificial insemination (AI) was performed using semen from the same bull of proven fertility 12 or 24 hours after estrous behavior was observed in the donor cow. Blastocysts were collected by routine non-surgical uterine method flushing seven days after the first AI. Finally, the blastocysts were picked out under a body-view microscope.

2.3. Test material handling

Blastocysts were placed individually into a micro drop of 50 µL of embryo biopsy medium on the surface of a 30 mm petri dish. The puncture needle (inner diameter of 20 µm) fixed on the micromanipulator was gently inserted into the middle of the embryo held by the fixation needle (Outer diameter 100 µm, inner diameter 25 µm). Then five to eight internal cells were aspirated

and transferred to a 1.0-mL sterile centrifuge tube, for sex determination. Another portion was directly placed into a 0.2-mL nuclease-free PCR tube and stored at −80 °C until processing for scRNA-seq analysis.

2.4. Sex determination of embryos by nested PCR

A built-in control nested PCR technique based on the amelogenin (*AMEL*) gene amplification was used. The bovine *AMEL* gene consists of six exons and five introns. It is located on the sex specific region of both the bovine X (AMELX) and Y (AMELY) chromosomes. Among them, there are about 63 nucleic acid deletions in the Y chromosome copy of the gene, which is a good tool for sex identification (Das et al., 2019). Briefly, we designed specific primers (Table 1) by referring to the cDNA sequence of the bovine *AMEL* gene (Accession numbers: NM_001014984 (*AMELX*) and NM_174240 (*AMELY*)) in GenBank. For nested PCR, 20 µL of PCR master mix containing 10 µL 2x Ultra Taq PCR Mix (containing Hot Star Taq DNA Polymerase, Multiplex PCR Buffer, dNTP mix), 4.6 µL DNase-free H_2O, 6 µL DNase-free H_2O, 0.4 µL of 10^{-2} mM concentration of forward and reverse primer mix, and 5 µL of genomic DNA (About 10^{-2} ng). The PCR rounds with each set of outer primers were performed on a Bio-Rad CXF96 PCR thermocycler (Bio-Rad, Hercules, CA, USA) with one cycle of 95 °C for 3 min, 39 cycles of 95 °C for the 30 s, annealing temperatures of 62 °C for 20 s, and 72 °C for 45 s, and a final extension step of 72 °C for 5 min. A nested PCR was performed with inner primers using the initial PCR product as a template. The second PCR was cycled under the same conditions as the initial PCR. Products were visualized on 2% (W/V) agarose gel. The gel was incubated with ethidium bromide (EB) at a final concentration of 0.5 µg/mL and visualized under ultraviolet illumination using a gel imaging system (Hisense Kelon model GelDoc 2000, Shanghai, China). Trans DNA Marker I is used as a gel electrophoresis marker (TransGen Biotech Co., Ltd., Beijing, China). The electrophoresis results showed that a single fragment of 329 bp was assigned as female and two fragments of 329 bp and 266 bp were considered male.

Table 1 - Parameters of primer pairs for the *AMEL* gene

Primer name		Primer sequence	Tm (°C)
Outer	*AMEL*-F1	5'-CATGGTGCCAGCTCAGCAG-3'	62
	AMEL-R1	5'-CCGCTTGGTCTTGTCTGTTGC-3'	
Inner	*AMEL*-F2	5'-CAGCAACCAATGATGCCAGTTC-3'	62
	AMEL-R2	5'-GTCTTGTCTGTTGCTGGCCA-3'	

Tm - annealing temperature.

2.5. Library preparation and sequencing

Three female blastocysts (as the treatment group, denoted as BLX) and three male blastocysts (as the control group, denoted as BLY) were chosen for further treatment. Individual blastocysts were transferred into 10-µL micro drops of streptomycin (PE, final concentration 5 mg/mL) using a mouth capillary tube and then incubated at 50 °C for 0.5 to 2 min until the zona pellucida was dissolved. The embryos were then rinsed again with PBS (5% FBS + 2% PS). Embryos were placed into 200-µL nuclease-free PCR tubes containing 10-µL single-cell lysate provided by Beijing Annoroad Gene Technology Co., Ltd. The single-cell lysate contains cell lysis components and RNase inhibitors, and the nucleic acid sequence with Oligo dT is used for reverse transcription to form the 1st cDNA. After the cDNA was amplified by Smart-Seq2 technology, the transcriptome sequencing library was constructed. The Qubit® 3.0 Fluorometer was used to detect the concentration of cDNA amplified by Smart-Seq2. Agilent 2100 High Sensitivity DNA Assay Kit was used to detect the fragment distribution of amplified cDNA samples.

For each sample, 40 ng of amplified cDNA was used as the starting material for library construction. The sample cDNA was interrupted by ultrasound, and the fragment size was about 350 bp. The fragment underwent end repair, "A" addition, and adapter addition. At the same time, to introduce different index tags, the adapter products were also subjected to PCR amplification. Library fragments in the range of 380-480 bp were recovered using a fully automated high-throughput fragment recovery system to complete the final library construction. The constructed library was sequenced using the Illumina Hiseq 6000 platform (Illumina, San Diego, CA, USA) for 150 bp paired-end sequencing (PE150).

2.6. Sequencing data analysis

In this study, transcript libraries from female and male blastocysts produced *in vivo* in bovine were established and analyzed by scRNA-seq. Quality control and data filtering were performed using the program Trimomatic (version 0.36; parameter: SLIDINGWINDOW:4:25; MINLEN:30; LEADING:3; TRAILING:3) (Bolger et al., 2014). Then, the clean reads were mapped to the *Bos taurus* genome (UMD3.1.86) using HISAT2 (version 2.1.0; parameter: no-unal) (Pertea et al., 2016), and the resulting SAM files were converted to BAM format using samtools (version 1.2; parameter: s; b) (Talker et al., 2022; Yang et al., 2022). The expression level of each gene in each sample was calculated using FeatureCounts software (http://subread.sourceforge.net/). Gene expression levels were quantified using Fragments per Kilobase per Million Mapped Fragments (FPKM) (Florea et al., 2013). The FPKM method can eliminate the influence of differences in gene length and sequencing volume on the calculation of gene expression. The DEG between the treatment group (BLY) and the control group (BLX) were identified using DEseq2 (v1.22.2) (Love et al., 2014), with thresholds set at $|\log_2$ (Fold change)| ≥1 and $P<0.05$ to obtain up- or downregulated genes.

2.7. Gene ontology and pathway enrichment analysis of DEG

The G: GOSt function in G: Profiler (v. e106_eg53_p16_65fcd97) (Raudvere et al., 2019) was utilized to annotate gene function by alignment with databases including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). With $P<0.01$ adjusted as the significance threshold, significantly enriched GO items or pathways were found.

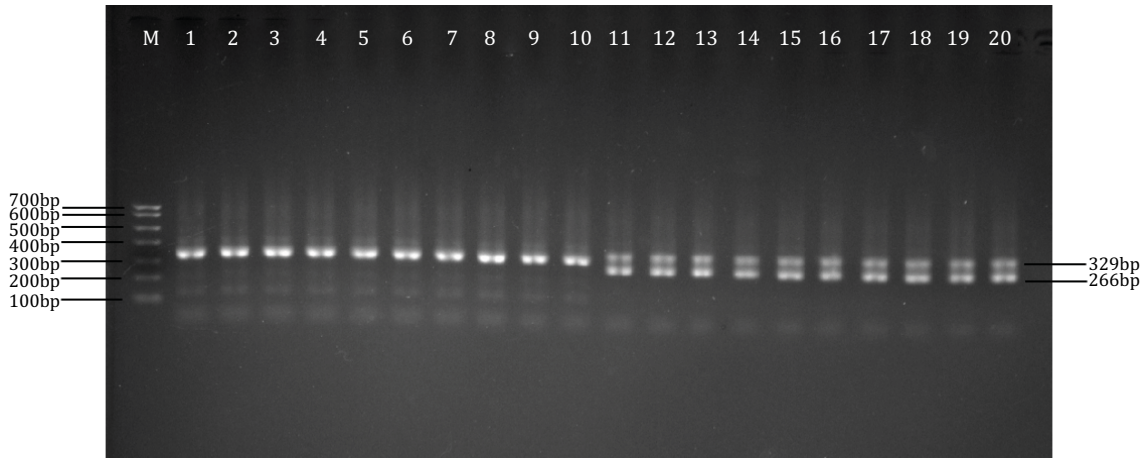
3. Results

3.1. Output from embryo collection and determination of embryo sex

All bovines reacted well to the superovulation protocol with normal-size ovaries and no unovulated follicles. A total of 24 blastocysts were collected, and sex was identified by nested amplification of the *AMEL* gene (Figure 1).

3.2. Sequencing data quality control and sequence alignment with reference genomes

After filtering out the sequences including joint pollution, low quality, and N content greater than 5%, clean reads accounted for more than 95.65% of raw reads. In clean reads, the percentage of Q30 base of each sample was > 91.79%, base distribution without AT, GC separation phenomenon, indicating good library quality. Clean reads of each library were aligned to the bovine genome, and the proportion of clean reads corresponding to the reference genome was between 95.45 and 96.33%, indicating that the effective data obtained by scRNA-Seq met the requirements of bioinformatics analysis (Table 2).



Total deoxyribonucleic acid was extracted from 5-8 internal cells of one blastocyst, followed by nested PCR amplification of the *AMEL* gene, and finally detected by 2% agarose gel electrophoresis.
M is the marker lane; lanes 1-10 are female blastocysts; and lanes 11-20 are male blastocysts.

Figure 1 - Nested PCR amplification results of the *AMEL* gene.

Table 2 - Summary of reading numbers in male and female blastocyst groups

Sample	Index				
	Raw read (n)	Clean read (n)	Clean read rate (%)	Clean Q30 base rate (%)	Mapping rate (%)
BLX_1	50,118,680	48,724,936	97.22	92.04	96.09
BLX_2	49,762,856	448,152,796	96.76	91.93	95.73
BLX_3	49,779,336	47,983,652	96.39	92.02	96.33
BLY_1	46,113,668	44,106,250	95.65	91.79	96.26
BLY_2	54,097,970	52,424,983	96.91	91.84	95.45
BLY_3	52,456,946	51,007,398	97.23	92.37	96.04

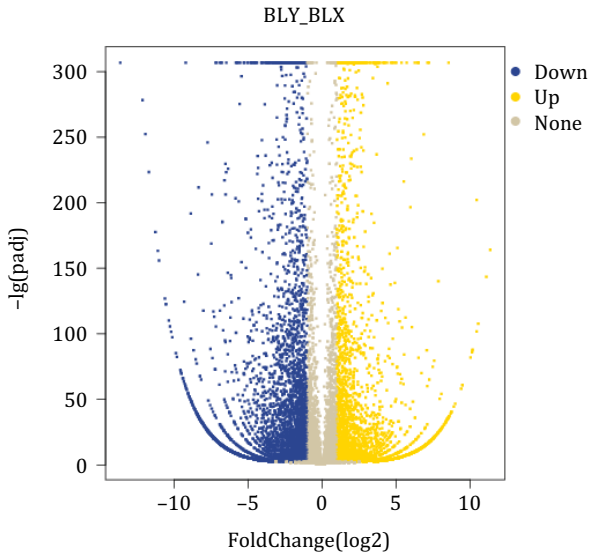
3.3. Identification of DEG among male and female blastocyst

In the comparison of the bovine female and male blastocyst, 8004 genes exhibited a significant difference in their expression levels with a threshold of $P<0.05$ and $|\log_2$ (Fold change) $|\geq 1$, including 3102 upregulated genes in BLY and 4902 downregulated genes in BLX (Figure 2).

3.4. GO functional enrichment analysis of DEG

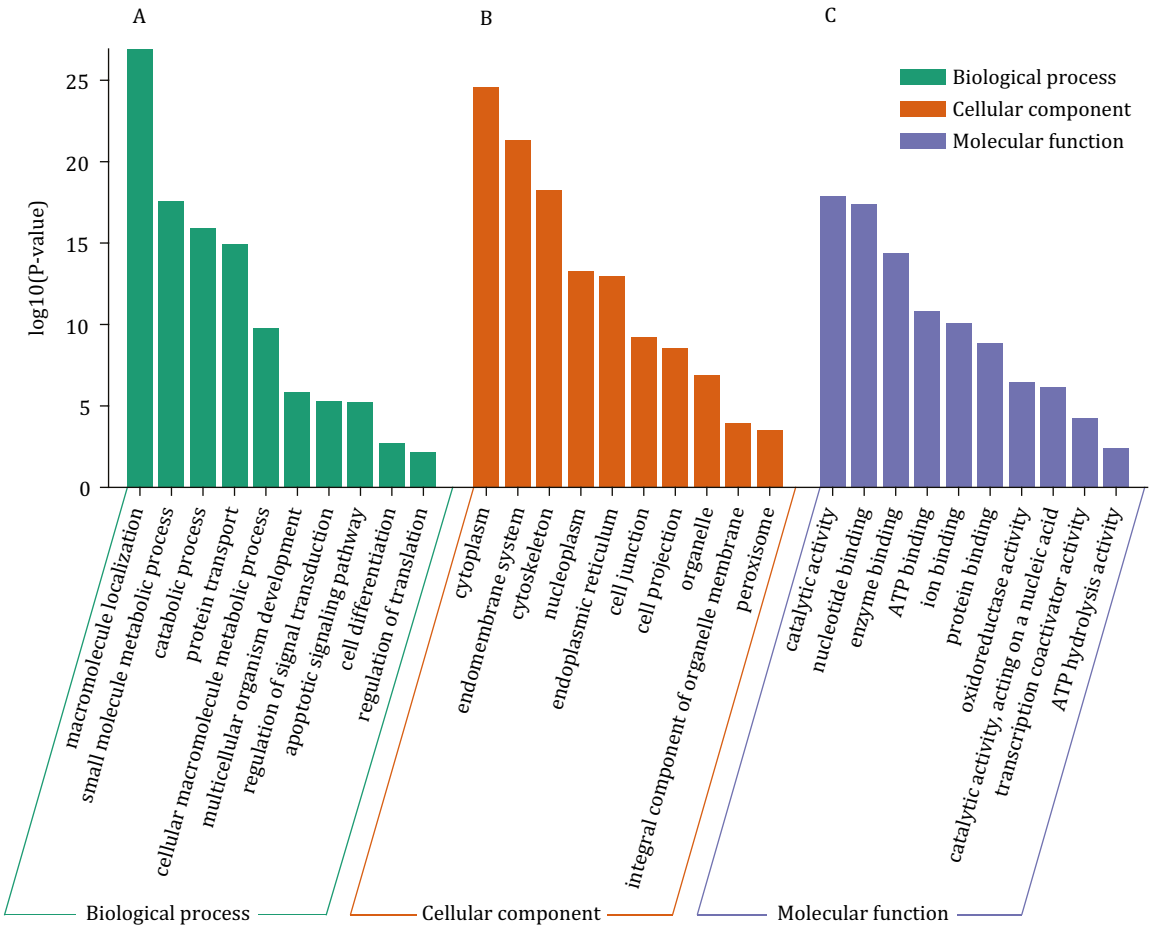
3.4.1. GO enrichment analysis of the upregulated genes in the female blastocyst

The screened upregulated genes in the female blastocyst were annotated to the GO database. The GO terms of the DEG were classified into three categories (58 terms): annotated by 28 biological processes, 13 cellular components, and 17 molecular functions. The top 10 gene ontology terms in each classification were counted (Figure 3). It was found that among the upregulated genes in the female blastocyst, catabolic processes, multicellular organism development, regulation of signal transduction, and apoptotic signaling pathway were significantly enriched. Cellular component analysis showed that cytoplasm, endomembrane system, cytoskeleton, endoplasmic reticulum, cell projection, and so on were overrepresented. In addition, under the molecular function, catalytic activity, nucleotide binding, enzyme binding, ion binding, and transcription coactivator activity were significantly enriched.



Yellow represents up-regulated genes in the BLY group, blue represents down-regulated genes in the BLX group, and gray represents insignificant differences in the two groups. X-axis represents Fold Change(log2); Y-axis represents -lg(padj).

Figure 2 - Volcano map showing differentially expressed genes (DEG) among female and male blastocysts.

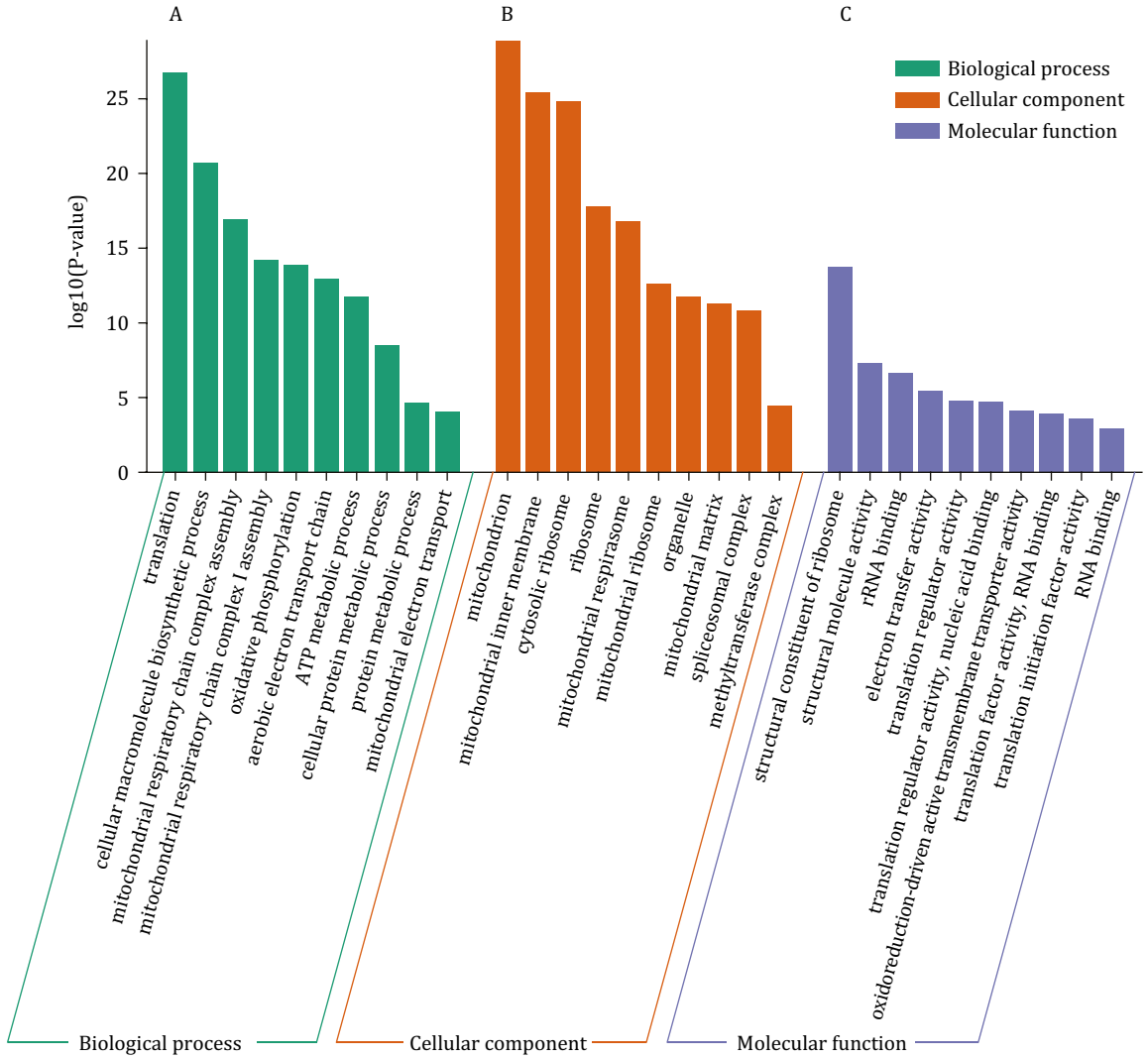


A: Top 10 biological processes; B: Top 10 cellular components; C: Top 10 molecular functions.
The Y axis is -log10 (P-value), in which the numerical value represents the probability of a false positive rate in the test.

Figure 3 - Gene ontology enrichment analysis of the upregulated differentially expressed genes (n = 4902) in female blastocyst.

3.4.2. GO enrichment analysis of the upregulated genes in the male blastocyst

The GO terms of the upregulated DEG in males were classified into three categories (53 terms): including 25 biological processes, 15 cellular components, and 13 molecular functions. The top 10 GO terms in each category were counted (Figure 4). The abundant terms in the biological process category were translation, mitochondrial respiratory chain, oxidative phosphorylation, and ATP metabolic process. In the cellular component category, DEG were mainly distributed in terms of the mitochondrion, mitochondrial inner membrane, ribosome, and spliceosomal complex. Structural constituents of the ribosome, structural molecule activity, ribosomal RNA (rRNA) binding, and translation regulator activity were significant enrichment terms in the molecular function category.



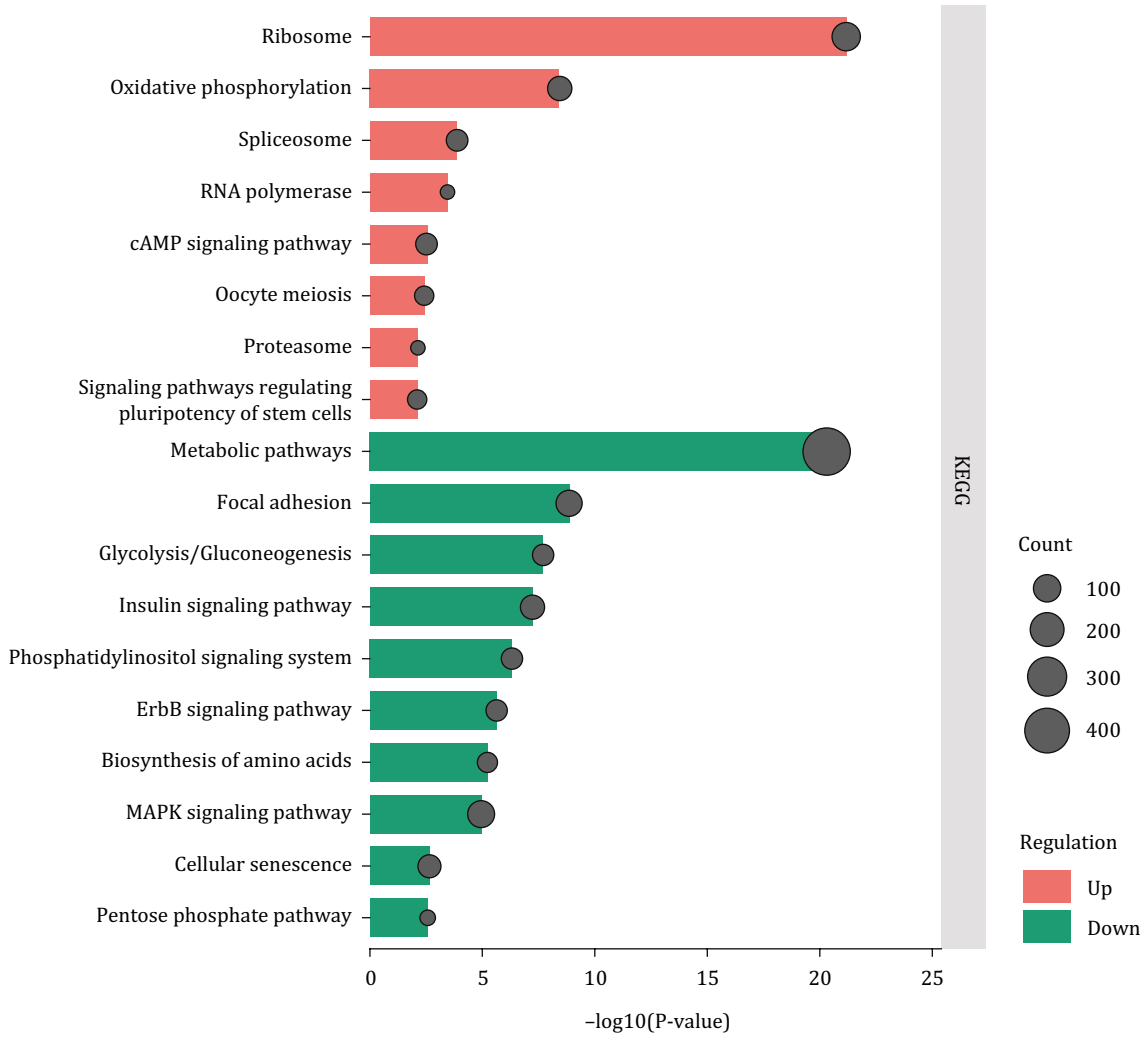
A: Top 10 biological processes; B: Top 10 cellular components; C: Top 10 molecular functions.
The Y axis is -log10 (P-value), in which the numerical value represents the probability of a false positive rate in the test.

Figure 4 - Gene ontology enrichment analysis of the upregulated differentially expressed genes (n = 3102) in male blastocyst.

3.5. KEGG functional enrichment analysis of DEG

The DEG were annotated to the KEGG database. Eight upregulated pathways in BLY and ten downregulated pathways in BLX were counted after P<0.01 correction during the male and female

blastocyst groups (Figure 5). The upregulated genes in the male blastocyst were annotated in the ribosome, oxidative phosphorylation, spliceosome, and other pathways. Metabolic pathways, focal adhesion, glycolysis, and cellular senescence pathways significantly enriched the upregulated genes in female blastocysts.



Top 18 enriched KEGG pathways in male and female blastocyst groups.
The X axis is -log10 (P-value), in which the numerical value represents the probability of a false positive rate in the test.
The size of different circles indicates the number of enriched genes (100, 200, 300, and 400). Red and green histograms show pathways enriched in upregulated genes in the male blastocyst group and upregulated genes in the female blastocyst group, respectively.

Figure 5 - Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of 8004 differentially expressed genes.

3.6. DEG involved in blastocyst growth and development

Several genes related to male and female blastocyst development were identified (Table 3). These include: Glucose-6-Phosphate Dehydrogenase (*G6PD*) (P = 1.80E-06), Hypoxanthine Phosphoribosyltransferase 1 (*HPRT1*) (P = 4.94E-14), Four and A Half LIM Domains 2 (*FHL2*) (P = 2.68E-37), and Calpain 6 (*CAPN6*) (P = 2.57E-27), which are significantly upregulated in female blastocysts; DNA Methyltransferase 3 Alpha (*DNMT3A*) (P = 1.10E-05), Growth Arrest and DNA Damage Inducible Gamma (*GADD45G*) (P = 3.83E-09), and Mitochondrial Ribosomal Protein L1 (*MRPL1*) (P = 0.02444), which are upregulated in male blastocysts; and Fructose-Bisphosphatase 1 (*FBP1*) (P =1.91E-15), which is specifically expressed in female blastocysts, and Solute Carrier Family 22 Member 14 (*SLC22A14*) (P = 2.13E-47), which is specifically expressed in male blastocysts (Table 4).

Table 3 - Developmentally related differentially expressed genes in female and male blastocyst groups

Gene ID	Gene name	Function description
ENSBTAG00000019512	*G6PD*	Involved in controlling the number of oxygen radicals.
ENSBTAG00000046220	*HPRT1*	Involved in controlling the number of oxygen radicals.
ENSBTAG00000001086	*FHL2*	*FHL2* acts as a transcriptional co-activator of *WT1* and regulates early gonadal differentiation to develop into testes or ovaries.
ENSBTAG00000000828	*CAPN6*	Participate in cytoskeletal remodeling processes, cell differentiation, apoptosis, and signal transduction.
ENSBTAG00000009733	*FBP1*	Inhibits cell proliferation, invasion, and migration.
ENSBTAG00000021143	*DNMT3A*	*DNMT3A* has been linked to sex-related epigenetic differences in terms of DNA methylation and telomere length.
ENSBTAG00000003033	*GADD45G*	Plays a role in gene activation by promoting DNA demethylation and MAPK signaling.
ENSBTAG00000018360	*MRPL1*	Mitochondrial ribosomal proteins (MRP) are essential components for the structural and functional integrity of the mitoribosome complex.
ENSBTAG00000008463	*SLC22A14*	*SLC22A14* has been characterized as an organic cation transporter-like protein and is one of the candidate genes for male fertility.

Table 4 - Developmentally related differentially expressed genes in female and male blastocyst groups

Gene ID	Gene name	Log2FoldChange	BLY _ BLX	P-value
ENSBTAG00000001086	*FHL2*	−8.34	Down	2.68E-37
ENSBTAG00000000828	*CAPN6*	−3.53	Down	2.57E-27
ENSBTAG00000046220	*HPRT1*	−6.57	Down	4.94E-14
ENSBTAG00000019512	*G6PD*	−2.36	Down	1.80E-06
ENSBTAG00000009733	*FBP1*	−6.74	Down	1.91E-15
ENSBTAG00000021143	*DNMT3A*	2.25	Up	1.10E-05
ENSBTAG00000018360	*MRPL1*	1.95	Up	0.02444
ENSBTAG00000003033	*GADD45G*	5.77	Up	3.83E-09
ENSBTAG00000008463	*SLC22A14*	9.01	Up	2.13E-47

4. Discussion

In the past 10 years, the annual per capita consumption of meat products in China has increased from more than 30 kg to more than 60 kg. It is particularly necessary to solve the practical problems of the number and output of livestock and poultry in animal husbandry, and to use the sex difference mechanism to achieve sex control for the development of animal husbandry. Studying the differences in mRNA levels between female and male blastocysts in bovines is important for exploring the sex-related molecular regulatory mechanisms of pre-implantation embryos. The present study filtered, assembled, and compared the transcriptome data of single male and female bovine blastocysts from *in vivo*-produced sources. Differentially expressed genes were annotated and functionally classified through bioinformatics analysis. Finally, we screened several genes related to embryonic development by GO terms and KEGG pathways. Thus, the possible mechanism of the difference between male and female embryonic development is revealed more accurately at the overall molecular level.

It has been noted that *in vitro*-produced bovine male embryos have faster development than female embryos (Sidrat et al., 2020). Gene ontology analysis was consistent with the observations reported. Among the molecular function, catalytic activity, nucleotide binding, ion binding, and transcription coactivator activity were significantly enriched in upregulated genes in female blastocysts, suggesting

a higher global transcriptional level in females. This is similar to the sex-specific methylation pattern that is high in male blastocysts and low in female blastocysts (Gebert et al., 2009). According to reports, during the pre-implantation stage, efficient mitochondrial activity is a factor that determines the development rate between the sexes (Yang et al., 2018), at the same time, higher mitochondrial DNA copy numbers were found in male embryos (Zolini et al., 2020). This is consistent with the significant enrichment of GO items such as mitochondria, mitochondrial inner membrane, cytosolic ribosome, the mitochondrial respiratory, and mitochondrial matrix in male blastocysts. Conversely, in the upregulated genes in female blastocysts, small molecule metabolic process, catabolic process, multicellular organism development, and cellular macromolecule metabolic process were overrepresented, suggesting a more active metabolism. These results correlate with earlier literature showing an association between high embryonic developmental strength and lower metabolic levels (Leese et al., 2007). Our results also show that the genes upregulated in female blastocysts are significantly enriched in apoptosis signaling pathways and cell differentiation of GO items; these observations are similar to those previously reported (Ghys et al., 2016). Finally, ribosomes, protein metabolic process, translation regulator activity, and others were significantly enriched in male blastocyst upregulated genes, indicating more active protein metabolism, which is associated with differences in amino acid biosynthesis between male and female bovine embryos (Sturmey et al., 2010; Green et al., 2016).

One of the pathways significantly enriched in the upregulated female genes is the pentose phosphate pathway, which plays a role in controlling the number of oxygen radicals, which are not only involved in cellular damage mechanisms but also have a growth-stimulating effect (Peippo and Bredbacka, 1995). The activity of the pentose phosphate pathway has been reported to be four times higher in females than in male blastocysts in bovine embryos cultured *in vitro* (Bermejo-Álvarez et al., 2008), and a similar metabolism has been found in human blastocyst stage embryos (Huang et al., 2017). Oxygen radicals in female blastocysts may play a delaying role during development due to the highly reactive pentose phosphate pathway in female blastocysts. In addition, two genes involved in the control of oxygen radicals, glucose-6-phosphate dehydrogenase (*G6PD*) and hypoxanthine phosphoribosyl transferase (*HPRT1*), located on the X chromosome (Gutierrez-Adan et al., 2000), were found to be highly expressed in female blastocysts in our study. Thus, male blastocysts with appropriate levels of oxygen radicals have faster development than female blastocysts. Glycolysis is the major glucose-consuming pathway, responsible for energy and metabolite production in most living organisms (Strikoudis et al., 2016). Fructose-Bisphosphatase 1 (*FBP1*) is the rate-limiting enzyme in gluconeogenesis and maintains the balance between fructose-1,6-bisphosphate and fructose-6-phosphate during glycolysis (Jin et al., 2017). Some experiments have confirmed that overexpression of the *FBP1* gene can inhibit cell proliferation, colony formation, and migration (Yang et al., 2020; He et al., 2021), and its specific expression in female blastocysts may be one of the reasons for the slow development of female blastocysts. Four and a Half LIM Domains 2 (*FHL2*) acts as a transcriptional co-activator of Wilms tumor gene 1 (*WT1*) and regulates early gonadal differentiation to develop into testes or ovaries (Du et al., 2002). Overexpression of *FHL2* can significantly reduce the viability of sheep ovary granulosa cells and inhibit cell proliferation (Zhang et al., 2020). The expression of this gene is increased in female blastocysts when compared with male blastocysts. This may be a contributing factor to the slower development observed in female blastocysts.

Mitochondria play an important role in providing energy to the embryo (Mittwoch, 2004). Mitochondrial ribosomal proteins 1 (*MRPL1*) are essential components for the structural and functional integrity of the mitoribosome complex (Cheong et al., 2020). The expression of *MRPL1* is upregulated in male bovine blastocysts compared with female bovine blastocysts, which may play a role in the differences in development between male and female embryos. *DNMT3A* has been upregulated in male bovine blastocysts compared with female bovine blastocysts, which is consistent with previous reports (Bermejo-Álvarez et al., 2008). Experiments with animals such as mice and zebrafish have proven that this transferase has a great influence on the growth and development of embryos (Pastor et al., 2016). *GADD45G* is one of the three members of the growth arrest and DNA damage-inducible protein 45 gene families and mediates a variety of cellular processes, including

apoptosis, cell cycle arrest, and senescence (Liebermann et al., 2011). Male mice defective in the *GADD45G* gene can result in varying degrees of sex developmental disorders (Johnen et al., 2013). There are no reports of *GADD45G* gene deletion causing sexual developmental disorders in cattle, but the results of studies in mice all suggest that the *GADD45G* gene is an upstream activator of the sex-determining region of the Y chromosome (*SRY*) (Warr et al., 2022). *SLC22A14* has been characterized as an organic cation transporter-like protein and is one of the candidate genes for male fertility (Runkel et al., 2008). The expression of the *SLC22A14* gene will affect the production of ATP and reactive oxygen species (ROS), while mitochondria are the main source of cellular energy and ROS (Kuang et al., 2021), and its specific expression in male blastocysts may be related to the developmental differences between male and female embryos.

Understanding the molecular differences between sexes through sex control technology is of great significance to the development of animal husbandry (Zhang et al., 2018). First of all, sex control technology can give full play to the growth rate and meat performance of male animals, as well as the reproductive and lactation performance of female animals, thus obtaining huge economic benefits. Secondly, it can enhance the intensity of good traits, speed up the breeding process, and reduce the breeding cost, resulting in maximum genetic progress. In addition, the phenomenon of twin infertility can be overcome by controlling the sex of offspring, and the harm of sex-related harmful genes can be eliminated (Peng et al., 2023). At present, sex control technology is gradually improving, and many existing problems have been solved. However, there are still some technical issues that hinder the development of animal husbandry. Polymerase chain reaction is an *in vitro* amplification technique. In embryo sex identification, sex-specific genes on the X or Y chromosomes are usually selected for amplification, and the embryo's sex is determined according to whether specific genes can be amplified. At present, the nested PCR method for embryo identification is the most widely used (Italiya et al., 2023).

5. Conclusions

In terms of mRNA expression, genes such as *G6PD*, *HPRT1*, *FHL2*, *CAPN6*, *FBP1*, *DNMT3A*, *GADD45G*, *MRPL1*, and *SLC22A14* were significantly different in female and male bovine embryos, suggesting that these may be key genes that directly or indirectly contribute to the faster development of male embryos compared with female embryos. These results provide a reference for further research on the mechanism of developmental differences between male and female embryos. Similarly, there are some other possible problems in this study. We only screened the differentially expressed genes in the development of male and female embryos, and further research should be aimed at these genes to verify the accuracy of the expression level; secondly, a deeper study on the mechanism of these differentially expressed genes should be carried out around their biological function.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Q. Gao. Data curation: B. Cui. Formal analysis: B. Cui. Funding acquisition: Q. Gao. Investigation: B. Cui and J. Wang. Methodology: B. Cui, F. Huang and N. Li. Project administration: Q. Gao. Resources: F. Huang and N. Li. Visualization: B. Cui and J. Wang. Writing – original draft: B. Cui. Writing – review & editing: B. Cui, J. Wang, F. Huang and N. Li.

Acknowledgments

The authors would like to thank Di Fang for his assistance in data generation and Dr. Zhishuai Zhang and Dr. Zhiyuan Sui for their help with experimental design and manuscript preparation.

We thank for the project support of the Study on the Molecular Mechanism of the Bovine SRY Gene Promoting the Proliferation and Differentiation of Male Cleavage Embryos (Grant No. 31660657).

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